EXPRESSION AND T CELL RECOGNITION OF HYBRID ANTIGENS WITH AMINO-TERMINAL DOMAINS ENCODED BY Qa-2 REGION OF MAJOR HISTOCOMPATIBILITY COMPLEX AND CARBOXYL TERMINI OF TRANSPLANTATION ANTIGENS

By IWONA STROYNOWSKI, JAMES FORMAN,* ROBERT S. GOODENOW, SUSAN G. SCHIFFER, MINNIE McMILLAN,‡ SUSAN O. SHARROW,§ DAVID H. SACHS,§ and LEROY HOOD

*From the Division of Biology, California Institute of Technology, Pasadena, California 91125; the *Department of Microbiology, University of Texas Health Science Center at Dallas, Dallas, Texas 75235; the ‡Department of Microbiology, University of Southern California School of Medicine, Los Angeles, California 90033; and the §Immunology Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20205*

The class I genes of the mouse are encoded in the major histocompatibility complex (MHC)[1] on chromosome 17. The classical transplantation antigens, K, D, and L, encoded by the H-2 region of the MHC, are expressed on most somatic cells and are used as restriction elements for the T cell cytotoxic destruction of virally infected or neoplastically transformed cells. In contrast, the Qa and Tla regions of the MHC encode a variety of class I molecules whose tissues of distribution are known only in a few cases and whose physiological functions are entirely unknown. Fewer than 10 Qa/Tla loci have been identified by classical serology (4–6). Their relationship to the functionally defined products is unclear. Serologically defined Qa/Tla genes, like the classical transplantation antigen genes, encode 37,000–45,000 dalton integral membrane glycoproteins that are associated noncovalently with a 12,000 dalton polypeptide, β_2-microglobulin. Each of the serologically defined Qa/Tla polypeptides has a distinct tissue distribution. For example, the TL antigens, encoded by certain Tla genes, are found only on thymocytes (7), some lymphoid tumors (8), and activated T cells (9). The Qa-2 antigen appears to be confined to hematopoietic tissues, T lymphocytes, and B lymphoblasts (4). The Qa-1 antigen has been reported to be expressed on all lymphoid cells in the spleen and preferentially expressed on a T lymphocyte subpopulation (5, 6). Some of the Qa/Tla proteins expressed on mature lymphocytes have been reported to give rise to H-2-unrestricted weak

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[1] *Abbreviations used in this paper:* FCS, fetal calf serum; FITC, fluorescein isothiocyanate; FMF, flow microfluorometry; HAT, hypoxanthine, aminopterin, thymidine; Ltk^+ and Ltk^- cells, mouse fibroblasts that are thymidine kinase–positive or –negative; MEM, minimum essential medium; MHC, major histocompatibility complex.

graft rejection responses and secondary T cell cytotoxicity reactions (1–3). The mechanism by which specific cytotoxic T cells recognize and kill Qa-bearing cells is unknown.

Recently, DNA clones cross-hybridizing to those of the K, D, and L genes were isolated, mapped to the MHC, and characterized (10–15). 10 class I genes were mapped to the Qa-2 region (Q1–10) of the inbred BALB/c mouse, and 18 others were assigned to the Tla region (14; D. Fisher, S. Hunt, and L. Hood, manuscript in preparation). To study protein products of these sequences, the genes were introduced into L cells by DNA-mediated gene transfer, and their cell surface expression was monitored with monoclonal antibodies specific for class I molecules (13, 16). Only a few gene products were detected by this assay and the serologically defined Qa-2 molecules were not conclusively identified. The remainder of the genes may encode the following classes of molecules: (*a*) proteins that do not react with the monoclonal antibodies used, (*b*) tissue-specific molecules that are not expressed on transfected fibroblasts or are expressed at very low levels, (*c*) secreted proteins, or (*d*) proteins confined to the cytoplasm. Alternatively, some of the unexpressed class I genes could be pseudogenes.

We were interested in studying the structure, expression, and T cell recognition properties of these molecules. For the initial analysis we chose the Q6 gene from the BALB/c Crgl ($Qa\text{-}2^a$) mouse (previously designated as gene 27.1), because its complete nucleotide sequence is known (10) and because it was negative in the transfection assay described above. This gene was proposed recently to be an allelic equivalent of the $H\text{-}2^b$ Q7 gene from C57BL/10 mouse (17). The BALB/c Q6 gene has an exon structure similar to that of the K, D, and L genes. Exon 1 encodes the leader peptide; exons 2–4 the three external domains $\alpha 1$, $\alpha 2$, and $\alpha 3$; exon 5 the transmembrane and a portion of the cytoplasmic domain; and exons 6–8 the remainder of the cytoplasmic region. The Q6 gene has a stop codon located at the 3′ end of exon 5, resulting in a cytoplasmic region of only three positively charged residues. Thus the Q6 polypeptide, if expressed, would be some 25 amino acid residues shorter than the typical transplantation antigen (see below).

To characterize the coding properties of the Q6 gene, we have constructed hybrid class I genes by splicing 5′ and 3′ portions of Q6 gene to the complementary ends of the K^d, D^d, and L^d genes. In some cases the hybrid class I genes were expressed on the cell surface after transfection into mouse fibroblasts. We report here serological, biochemical, and functional characterization of the hybrid gene products and define regions on the Q6 molecule that contain polymorphic determinants recognized by anti–class I monoclonal antibodies and anti-Qa-2 region–specific alloreactive cytotoxic T cells.

Materials and Methods

Materials. The Q6, L^d, D^d, and K^d genes were derived from clones λ27.1 (10), λ27.5 (18), c18.1 (11), and λ1-3 (13), respectively. Restriction enzymes were purchased from New England Biolabs; Bethesda Research Laboratories, Gaithersburg, MD; and Boehringer Mannheim Biochemicals, Indianapolis, IN. T4 DNA ligase was obtained from Boehringer Mannheim Biochemicals. DNA were propagated in *Escherichia coli* strain MC1061. Monoclonal antibodies have been previously described (19–21). D3.262 is a monoclonal antibody described elsewhere (22). The 66-4-8 antibody has been recently isolated (S.

Chatterjee-Das, D. Hernandez, and D. H. Sachs, manuscript in preparation). The B6.K1 anti-B6 and B6.K1 anti-B6.K2 antisera were kindly provided by Lorraine Flaherty, Department of Health, Albany, NY. Anti-H-2^k serum was produced by immunizing B10.D2 mice with B10.BR cells. Animals were bred in the University of Texas Health Science Center at Dallas colony or purchased from The Jackson Laboratory, Bar Harbor, ME.

Construction of Recombinant Class I Genes. Recombinant genes were constructed using standard molecular biology techniques (see Fig. 1). A 3 kb BglII-BamHI fragment of the Q6 gene derived from λ27.1 (10) and containing exons 1–3 and 926 basepairs (bp) of 5′ flanking region was cloned into the BamHI site of pBR322. The resulting plasmid, p1104.4, was linearized with BamHI enzyme, and 2.4 kb BamHI fragments from λ27.5 (17) or c18.1 (11), containing exons 4–8, were inserted, making Q6/L^d and Q6/D^d genes, respectively. To construct the hybrid Q6/K^d gene, we cleaved p1104.4 with BamHI and EcoRI and inserted a 3.7 kb BamHI-EcoRI K^d fragment containing exons 4–8 and derived from λ1-3 (13). The hybrid L^d/Q6 gene was constructed by insertion of a 2.6 kb BamHI fragment (exons 4–8) from λ27.1 into a unique BamHI site of plasmid 2001 described previously (23). The L^d/Q6 construct has ~6 kb of 5′ flanking region and ~3 kb of 3′ flanking region. The identity of all the constructs was verified by extensive restriction enzyme analyses.

Transfection of Mouse Fibroblasts and Maintenance of Cell Lines. Mouse thymidine kinase–negative cells (Ltk^-) derived from C3H mice (H-2^k) were used as recipients in transfection experiments. They were maintained in alpha minimal essential medium (α-MEM) (Irvine Scientific, Santa Ana, CA) containing 10% fetal calf serum (FCS), glutamine, streptomycin, penicillin, and bromodeoxyuridine. Transfection was performed as previously described (24) using ~1 μg of plasmid or λ phage DNA, 20 ng of ptk5 (herpes simplex thymidine kinase gene cloned in pBR322), and 20 μg of Ltk^- carrier DNA per 5 × 10^6–10^7 Ltk^- cells. The Ltk^+ transfectants were selected in medium containing hypoxanthine, aminopterine, and thymidine (HAT).

Radioimmunoassay and Immunoprecipitations. Radioimmunoassays were performed as previously described (24). Briefly, an appropriate dilution of monoclonal hybridoma antibody was mixed with 5 × 10^5 trypsin-dispersed Ltk^+ cell transfectants, and incubated 4 h at 4°C in microtiter plates.The cells were washed twice and suspended in a medium containing ^{125}I–protein A (Amersham Corp., Arlington Heights, IL). The plates were incubated overnight at 4°C and the cells were washed twice and counted in Gamma 5500 counter (Beckman Instruments, Inc., Fullerton, CA). The experimental errors did not exceed 5%. For immunoprecipitations, the transfected cells were labeled with [^{3}H]-phenylalanine (New England Nuclear, Boston, MA; Amersham Corp.) as previously described (24, 25). Immunoprecipitated class I–like molecules were analyzed by two-dimensional gel electrophoresis (25).

Immunofluorescence Staining and Flow Microfluorometry (FMF) Analysis. Immunofluorescence staining was performed as previously described (26). For indirect staining, 6 × 10^5 cells, harvested by trypsinization, were incubated for 45 min at 4°C with monoclonal antibodies, washed twice, and incubated for 45 min with fluorescein (FITC)-goat isothiocyanate F(ab′)$_2$ anti–mouse IgG (60429; Boehringer Mannheim Biochemicals). After this incubation, cells were washed twice and resuspended for FMF analysis. For inhibition experiments, cells were incubated at 4°C for 60 min with the antibody being tested for blocking activity. After 60 min, fluorescein-conjugated antibody was added in limiting amounts and the incubation was continued for 20 min. After this incubation, cells were washed twice, resuspended, and analyzed for fluorescence.

FMF analysis was performed as previously described (26) using a FACS II (B-D Automated Immunochemistry, Becton, Dickinson & Co., Salt Lake City, UT). Fluorescence data were collected using logarithmic amplification on 50,000 viable cells as determined by forward light scattered intensity. Logarithmic amplification was provided by a 3-decade logarithmic amplifier constructed from an NIH modified design of R. Hiebert, Los Alamos Scientific Laboratory (Los Alamos, NM). Data are displayed as immunofluorescence profiles in which logarithmically increasing fluorescence intensity is

plotted on the *x* axis and cell number is shown on the *y* axis. Logarithmic values of median fluorescence intensity were converted to linear units of V (volts × 10^4) using a calibration curve generated on the amplifier used. Data from the inhibition experiments were calculated using the formula: percent inhibition = $100 - [(V_{exp} - V_{bkg})/(V_{con} - V_{bkg})]$, where V is linear units of mean fluorescence intensity; exp, cells stained with FITC-antibody in the presence of a potential inhibitor; con, cells stained with FITC-antibody in the presence of media; and bkg, unstained cells.

Generation and Assay of Cytotoxic T Cells. Anti-H-2 and anti-Qa-2 region–specific cytotoxic T cells were generated as described previously (11, 22). Briefly, anti-Qa-2 cytotoxic T cells were generated by priming B6.K1 (Qa-2^b) mice in vivo with A.BY (Qa-2^a) spleen cells, followed by in vitro challenge with (Qa-2^a) B6.K2 congenic cells. The mice were challenged in vitro no sooner than 4 wk after priming. The ^{51}Cr assay against L cells, including inhibition with antibody, has been described (27). Data are presented as net release of isotope from target cells. Net release equals percent release of isotope from target cells in the presence of effector cells minus spontaneous release. Spontaneous release from L cells was between 10 and 20%, and from B6.K2 target cells, between 25 and 35%. The standard error of the mean of triplicate samples did not exceed 2%.

A number of controls for the experiments described in Fig. 4 were carried out. First, to eliminate the possibility that, in the B6.K1 anti-B6.K2 sensitization, anti-H-2^k cytotoxic T cells are generated that preferentially lyse Q6/L^d-expressing cells, we crossed B6.K1 animals to C3H (H-2^k) animals and tested the (B6.K1 × C3H)F_1 offspring for their ability to generate cytotoxic T cells that would lyse Q6/L^d cells. This experiment is important because the mouse L cells are of the H-2^k haplotype. The lytic activity of cytotoxic T cells generated in this experiment was comparable to that observed using parental B6.K1 cytotoxic T cells. Second, to confirm that the determinant recognized by cytotoxic T cells is localized on the α1-α2 domain contributed by the Q6 gene, and not on α3-β_2-microglobulin domain contributed by the L^d gene, we tested cells transfected with the hybrid Q6/D^d gene. As predicted, these cells were lysed by both types of anti-Qa-2 region cytotoxic T cells described above. Finally, to independently test the activity of anti-Qa-2 region cytotoxic T cells, we sensitized BALB/cBy (Qa-2^b) with BALB/cJ (Qa-2^a) spleen cells. The lytic activity of these cytotoxic T cells was less than that of the B6.K1 killer T cells described above. However, they did specifically lyse the Q6/L^d target cells (data not shown). These results taken together indicate that anti-Qa-2 region cytotoxic T cells recognize antigenic determinant(s) on the α1-α2 domain of Q6 hybrid gene products.

Results

Strategy for Testing Coding Properties of Q6 Gene. The Q6 gene appears to be functional by sequence criteria (see Discussion), yet its product is not detectable by cell binding radioimmunoassay after transfer into mouse fibroblasts. This could result from the use of the reagents not specific for Q6 product, from the lack of Q6 expression on the cell surface of transfectants, or both. In any case, the Q6 gene is, in this system, operationally nonfunctional. Our approach to the study of the Q6 protein is to construct recombinant Q6/L^d genes, transfer them into fibroblasts, and characterize the properties of Q6-encoded domains in the context of hybrid molecules. The rationale for constructing these hybrids is as follows. (a) The exon-intron structure of all class I genes is virtually identical. Thus, homologous sequences encoding different domains can be exchanged between the L^d and Q6 genes without introducing reading frameshifts or deletions. The same is also true for the K^d and D^d genes. (*b*) The external portions of class I molecules, α1 and α2, appear to fold together to generate a single α1-α2 domain, and the α3 region and β_2-microglobulin fold together to generate a second external domain (23, 28–30). This conformation is analogous

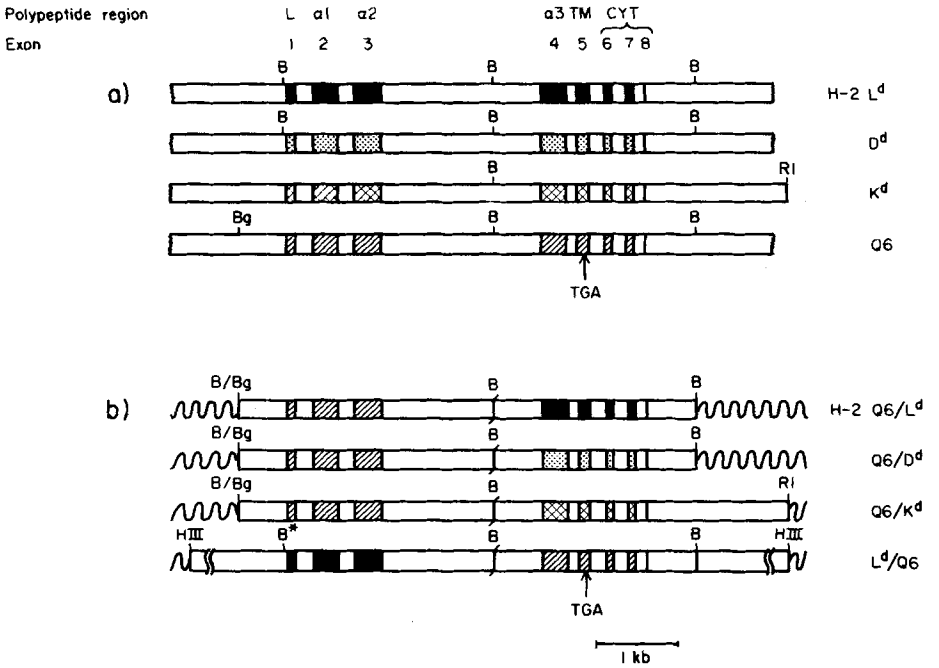

FIGURE 1. Exon-intron structure of (*a*) class I genes and (*b*) the recombinants. Exons are presented as filled boxes; 5′,3′ flanking regions and introns as open boxes. Waved lines correspond to pBR322 plasmid DNA into which the genes were cloned. Restriction sites: B, BamHI; Bg, BglII; RI, EcoRI; HIII, HindIII; B/Bg, hybrid site created by ligation of BamHI and BglII sticky ends and no longer recognized by either enzyme; B*, modified BamHI site no longer recognized by BamHI.

to the folding of the light and heavy chain variable (V_L and V_H) regions of immunoglobulins to form the V domain, and the folding of the L chain constant (C_L) and H chain constant (C_H)1 regions to form C_H1 domain (31). Thus, polymorphic determinants recognized by antibodies will generally be on the α1-α2 domain or α3-β_2-microglobulin domain, but not on both. Hence, the two distinct domains can be shuffled between different class I molecules (23, 28–30). The hybrid class I genes should join exons 1–3 (leader, α1, α2) from one gene with exons 4–8 from the second gene (α3, carboxyl terminus). Fig. 1 presents the structure of the recombinant class I genes constructed according to these assumptions. Two BamHI sites are conserved between Q6 and L^d sequences. One of them is located in the third intron, and the second is located downstream from the polyadenylation site. Hence, these sites can be used to construct hybrid Q6/L^d genes in which the leader, α1, and α2 regions are encoded by the Q6 gene, the remainder of the molecule by the L^d, and vice versa. The details of the hybrid gene constructions are presented in Materials and Methods. (*c*) Monoclonal antibodies have been identified, for the L^d gene products, which recognize the α1-α2 (30-5-7) or the α3-β_2-microglobulin (28-14-8) domains (23, 28). These two monoclonal reagents can be used to detect hybrid Q6/L^d (exons 1–3 from Q6 and exons 4–8 from L^d) gene product (reagent 28-14-8) or hybrid L^d/Q6 gene product (reagent 30-5-7). The properties of the Q6 portion of the

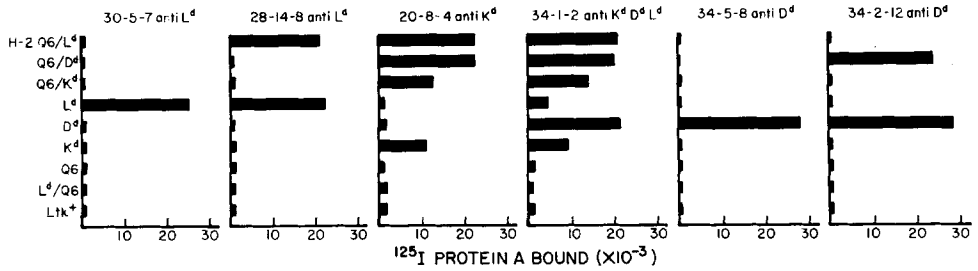

FIGURE 2. Results of cell binding radioimmunoassay on cloned L cells transfected with class I genes. The tests were carried out with 10^{-2} dilutions of ascites fluids or concentrated supernatants of hybridomas containing 30-5-7, 28-14-8, 20-8-4, 34-5-8, and 34-2-12 monoclonal antibodies. The numbers shown are the average of four experiments. The experimental errors are <10% of the values shown. K^d transfectant used in these experiments as control is an unstable line and expressed K^d molecules at low levels. All other transfectants were stable for one year when grown in α-MEM medium with HAT, glutamine, FCS, and antibiotics.

hybrid can be analyzed using different serological reagents and T cell cytotoxicity assays.

Hybrid Q6/L^d Gene Is Expressed on Mouse L Cells. The Q6/L^d gene was introduced into Ltk$^-$ fibroblast cells derived from C3H mice (H-2^k) by cotransfection with herpes virus thymidine kinase gene as previously described (12, 13). The Ltk$^+$ transfectants were tested by radioimmunoassay for the expression of hybrid Q6/L^d antigens using the 24-14-8 and 30-5-7 monoclonal antibodies that recognized the α3-β_2-microglobulin and α1-α2 domains of the L^d gene product, respectively (Fig. 2). The 28-14-8 reagent reacted with the Q6/L^d transfectants, but the 30-5-7 reagent did not. Neither antibody reacted with the untransformed mouse L cells. These observations suggested that a hybrid Q6/L^d class I molecule was expressed on the surface of the transfected mouse fibroblasts.

To determine whether the α1-α2 domain contributed by the Q6 gene had antigenic determinants crossreacting with monoclonal reagents to various transplantation antigens, we tested the Q6/L^d transfectants by radioimmunoassay using a variety of additional monoclonal reagents: 34-1-2 (anti-K^d, -D^d, -K^b, -L^d, -r, -s, -p, -q), 20-8-4 (anti-K^d, -K^b, -r, -s), 34-5-8 (anti-D^d), and 34-2-12 (anti-D^d). The 34-1-2 and 20-8-4 antibodies reacted with Q6/L^d-transfected cells but not with the recipient Ltk$^+$ cells (Fig. 2). The observed crossreactivities suggest that the Q6 α1-α2 domain contains antigenic determinants similar to those found on the classical transplantation antigens.

We also demonstrated that an alloantiserum specific for molecules encoded by the Qa-2 region (B6.K1 anti-B6.K2) reacted with Q6/L^d hybrid molecules (see below). This antiserum does not crossreact with any known determinants on transplantation antigens and, accordingly, this observation suggests that there are antigenic determinants on the α1-α2 domains of the hybrid Q6/L^d molecule that are not found on the classical transplantation antigens.

We have extended our serological analyses using FMF analysis. In direct typing experiments, mouse L cells transfected with Q6/L^d gene were positive with the 28-14-8, 20-8-4, and 34-1-2 antibodies, and negative with 30-5-7 (anti-L^d, -D^q, -L^q), 31-3-4 (anti-K^d), 34-5-8 (anti-D^d), 23B10.1 (anti-L^d, -D^q, -L^q), 28-13-3 (anti-

K^b, -f), 66-4-8 (anti-D^q, -D^d, -L^d, -D^b, -D^s, -f, -p, -r), D3.262 (anti-Qa-2^a), 28-8-6 (anti-K^b, -D^b), and 34-7-23 (anti-K^d, -D^d, -K^b, -q) reagents (data not shown).

To determine the molecular weight of the hybrid Q6/L^d gene product and to verify that the determinants recognized by the antibodies on Q6/L^d transfectants are expressed on the same polypeptide, monoclonal antibodies 20-8-4 (α1-α2 domain of Q6) and 28-14-8 (α3-β_2-microglobulin domain of L^d) were used independently to immunoprecipitate the Q6/L^d gene products (Fig. 3). In addition, we tested the transfected products by precipitation with an alloantiserum specific for molecules encoded in Qa-2 region (B6.K1 anti-B6.K2, data not shown). The immunoprecipitates were analyzed by two-dimensional gel electrophoresis and compared with class I molecules of the d haplotype precipitated from L cell transfectants. In each case, the Q6/L^d products precipitated in association with the ~12,000 mol wt polypeptide, β_2-microglobulin. The larger, 45,000 mol wt molecules display isoelectric heterogeneity characteristic of glycosylated class I products (13). The Q6/L^d polypeptides immunoprecipitated by each of these antisera are identical to each other and clearly different from L^d, K^d, or D^d polypeptides precipitated from L cell transfectants or BALB/c spleen (12, 13, 23). The control experiments showed that 28-14-8 and 20-8-4 monoclonal antibodies and B6.K1 anti-B6.K2 serum do not precipitate any molecules from the untransfected L cells. These experiments establish that Q6/L^d polypeptide is an ~45,000 mol wt protein which carries antigenic determinants characteristic of the transplantation antigens as well as Qa-2 molecules. It is expressed on the cell surface of the transfectants in association with β_2-microglobulin.

Antibodies Against K^d Molecules and Antigens Encoded by Qa-2 Region Detect Distinct but Spatially Related Determinants on the Q6/L^d Molecules. To locate relative positions of determinants recognized by 20-8-4, 34-1-2, and B6.K1 anti-B6 serum on the Q6/L^d molecules, we performed inhibition studies (Fig. 4). Binding of both 20-8-4 and 34-1-2 monoclonal antibodies to Q6/L^d products could be blocked by each other or by B6.K1 anti-B6.K2 serum. No inhibition was seen with L^d α3-β_2-microglobulin domain–specific antibodies positive in direct typing experiments (28-14-8), or with monoclonal antibodies negative in the direct typing experiments (34-5-8, D3.262). In contrast, the reactivity of K^d gene transfectants with 20-8-4 or 34-1-2 monoclonal antibodies could not be inhibited by B6.K1 anti-B6 serum. Clearly, the determinants detected by B6.K1 anti-B6 serum are absent from K^d transplantation antigens but present on the Q6/L^d epitope spatially related to 20-8-4- and 34-1-2 K^d-like determinants. This inhibition analysis has led us to propose that the Q6 gene may encode the serologically defined, Qa-2 region–specific antigen described previously on subpopulations of lymphocytes (see Discussion).

Q6/K^d and Q6/D^d Hybrid Genes Are Also Expressed on the Surface of Mouse L Cells. We have considered the possibility that the polymorphic determinants detected by 20-8-4 and 34-1-2 on the α1-α2 domain of Q6/L^d are created by the interaction of the α1-α2 domain of Q6 with the α3-β_2-microglobulin domain of L^d. To exclude this possibility, hybrid Q6/K^d and Q6/D^d genes were constructed (see Materials and Methods and Fig. 1) and transfected into mouse L cells as described above. Both hybrid genes express class I gene products detectable with the 20-8-4 and 34-1-2 antibodies (Fig. 2). In addition, the Q6/D^d transfected

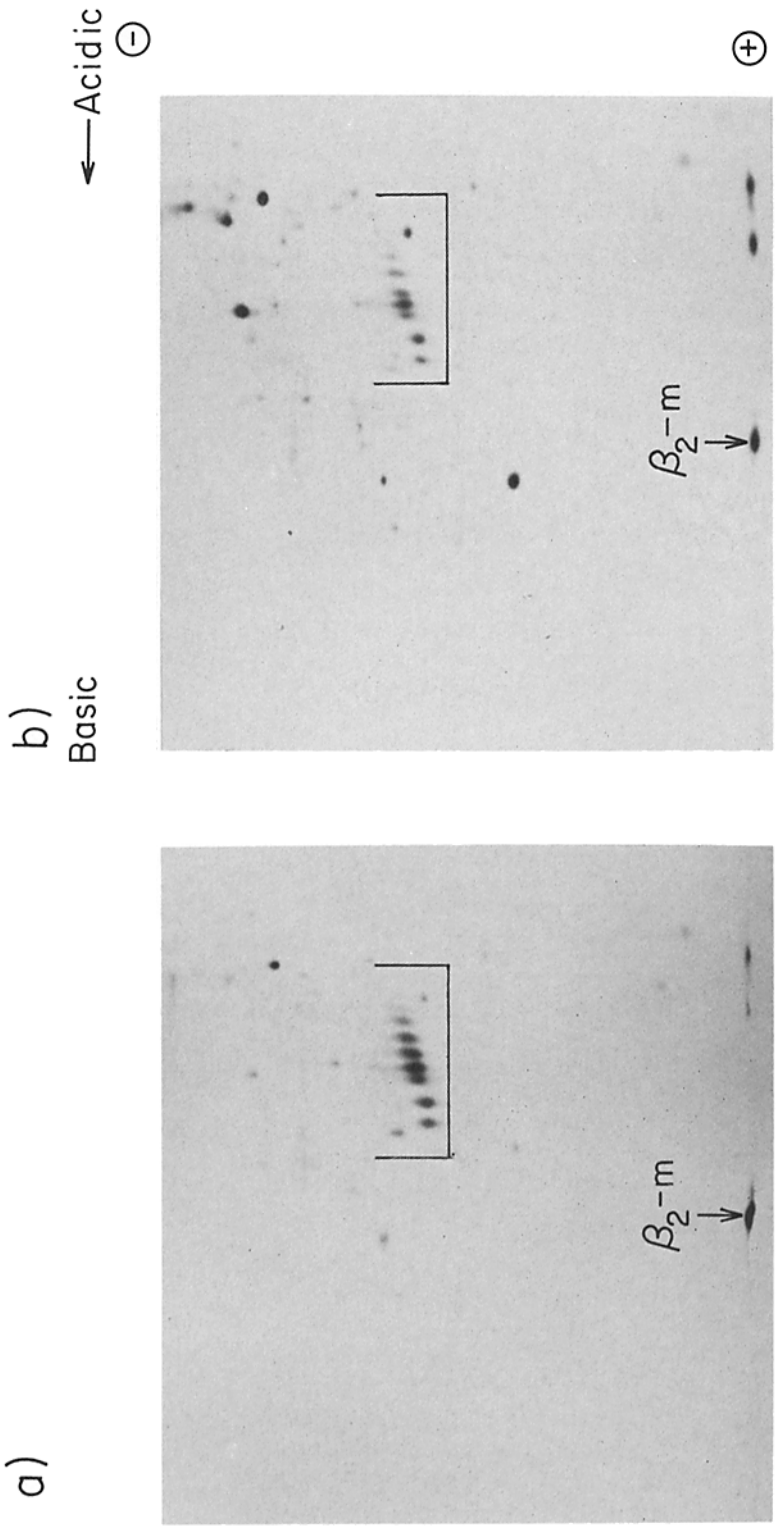

FIGURE 3. Fluorographs of two-dimensional gels of Q6/L^d molecules precipitated from cell lysates of clone-transfected fibroblasts. Monoclonal antibodies used in immunoprecipitates were (*a*) 20-8-4 and (*b*) 28-14-8. Spots corresponding to class I-like glycoproteins are boxed. The arrows point to β_2-microglobulin.

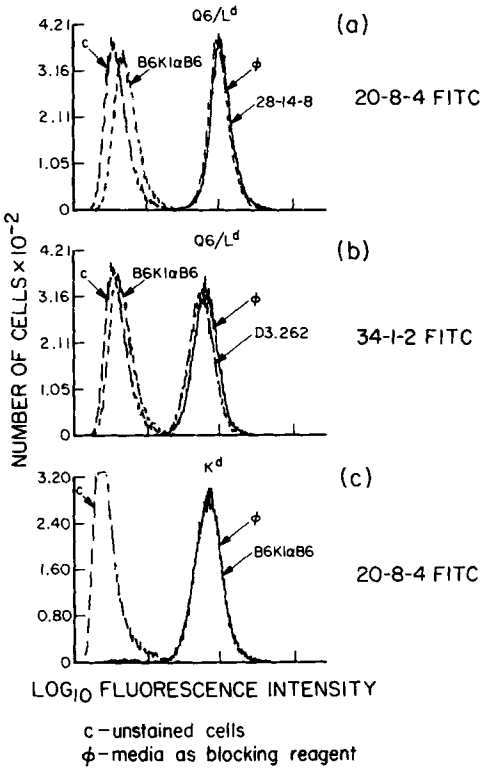

FIGURE 4. Analysis of serological determinants on Q6/L^d and K^d antigens by blocking with specific antibodies. Inhibition of binding of specific antibodies: 20-8-4 (*a*, *c*) and 34-1-2 (*b*) to L cells transfected with Q6/L^d (*a*, *b*) and K^d (*c*) genes. L cell transfectants were reacted with media or the following blocking reagent: 28-14-8 (anti-L^d), D3.262 (anti-Qa-2), or B6.KI anti-B6 sera (anti-TLa complex).

cells react with 34-2-12, a monoclonal antibody specific for the $\alpha 3$-β_2-microglobulin domain of the D^d molecule (23, 28). These results confirm that serological determinants detected on the $\alpha 1$-$\alpha 2$ domain are not dependent on the presence of a particular $\alpha 3$-β_2-microglobulin domain in the Q6 hybrids. These observations further support the hypothesis that these regions fold to constitute two distinct external domains.

Hybrid L^d/Q6 Polypeptides Are Not Expressed on Mouse L Cells. The Qa and Tla genes are expressed only in certain subpopulations of lymphocytes, in contrast to the transplantation antigens, which appear to be expressed on most somatic tissues. Accordingly, the L^d, K^d, and D^d genes are readily expressed in mouse L cells, whereas many Tla complex genes, including the Q6 gene, are not (13). One explanation for this behavior is that the Q6 gene encodes features which control tissue expression. Certainly the observation that the 5′ portion of the Q6 can be expressed at high levels as a Q6/K^d, Q6/D^d, or Q6/L^d hybrid gene is consistent with the notion that this feature is not contained in the 5′ portion of the Q6 sequence. Clonal analysis showed that >90% of the cells derived from the pool of Q6/L^d-transfected fibroblasts were positive for the

expression of Q6 determinants (data not shown). Thus, transcription from the chromosomal promoters located upstream of Q6/L^d integration sites appears unlikely, as such events would be expected to occur with low frequency. The possibility that the recombinant genes are transcribed from sequences within the bacterial plasmid was excluded by the analysis of deletion mutants lacking 5′ flanking regions (data not shown). We propose that the 5′ flanking region (926 nucleotides) upstream from the Q6 gene encodes a promoter that functions as a transcription initiation site in fibroblasts and that the 3′ portion of the gene is responsible for the lack of Q6 cell surface expression. Accordingly, we constructed an L^d/Q6 hybrid gene (5′ flanking sequence and exons 1–3 from L^d and exons 4–8, and 3′ flanking sequence including polyadenylation site, from Q6) (Fig. 1). This gene was introduced into L cells, and its expression was monitored by cell binding radioimmunoassay using monoclonal antibody 30-5-7 (specific for α1-α2 domain of L^d) (Fig. 2). In addition, intact Q6 gene transfectants were tested by immunoprecipitation of whole cell lysates and culture supernatants using monoclonal antibody 20-8-4 (specific for α1-α2 domain of Q6) (data not shown). No crossreacting class I gene products were detected in either case. Thus, the 2.6 kb BamHI fragment encoding the 3′ portion of the Q6 gene appears to contain a sequence that suppresses the expression of the 5′-coded determinants on the L^d and Q6 molecules. Different types of phenomena could be involved (see below). We are currently testing whether the 3′ end–encoded feature exerts its effect at the RNA or the protein level.

Cytotoxic T Cells Reactive With Qa-2 Region Antigens Recognize the α1-α2 Domain of Q6/L^d Molecule. The specific alloreactive cytotoxic T cells also can be used to characterize antigenic determinants on the hybrid Q6/L^d molecules. Forman et al. (22) have previously generated cytotoxic T lymphocytes by immunization of B6.K1 (Qa-2^b) mice with B6.K2 (Qa-2^a) spleen cells. The chromosomes of these two recombinant strains, B6.K1 and B6.K2, are identical except for the area defining the Qa-2 region (6). Therefore, the alloreactive cytotoxic T cells produced by this immunization would be expected to react against haplotype-specific determinants of class I molecules encoded by the Qa-2^a region and expressed on the spleen cells of B6.K2 mice. One serologically defined class I molecule, Qa-2, is known to be encoded by this region. It has been shown that monoclonal antibodies directed against the Qa-2 molecule block only partially the activity of the Qa-2 region–specific cytotoxic T cells (22). Therefore, these cytotoxic T cells may detect Qa-2 region–encoded specificities other than the Qa-2^a molecule. To test whether the Q6 molecules could serve as potential targets for alloreactive cytotoxic T cells, we tested T cells generated in the B6.K1 anti-B6.K2 combination on mouse L cells transfected with the hybrid Q6/L^d gene. Fig. 5 demonstrates that cytotoxic T cells specific for the Qa-2 region recognize and kill L cell transfectants expressing Q6/L^d molecules, but not the control Ltk^+ recipients or L cells transfected with the L^d gene. Thus the Q6/L^d gene product clearly expressed Qa-2 region–coded determinants that can readily be detected with Qa-2-specific alloreactive T cells. A number of controls were carried out to establish that the Qa-2-specific killer T cells were directed against determinants on the α1-α2 domain of the Q6/L^d gene product (see Materials and Methods). These results parallel recent findings by Reiss et al. (32), Stroy-

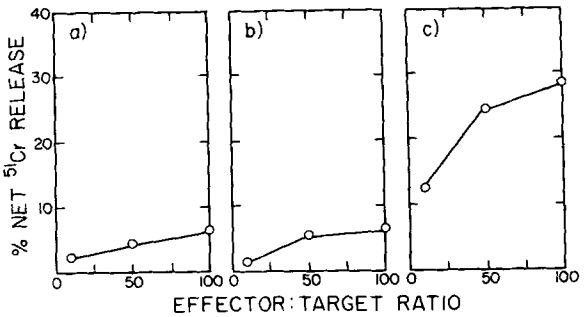

FIGURE 5. Recognition of Q6 hybrid proteins by cytotoxic T cells specific for Qa-2 region–coded molecules. Net release of ^{51}Cr from Ltk$^+$ cells: (*a*) H-2L^d cloned transfectants, (*b*) H-2Q6/L^d cloned transfectants. (*c*) Similar results were obtained on uncloned mass transfectants. B6.K1 anti-B6.K2-cytotoxic T lymphocytes were generated by in vivo priming and in vitro sensitization.

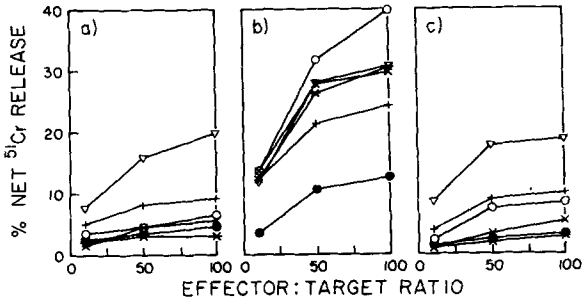

FIGURE 6. Sensitivity of cells transfected with Q6/L^d gene to anti-H-2 cytotoxic T cells. Sensitivity of L cell transfectants to anti-H-2 cytotoxic T cells is shown as net release of ^{51}Cr from Ltk (*a*) and L^d (*b*) and Q6/L^d (*c*) cloned transfected lines. The following primary cytotoxic T cells were tested with their lytic potential against the L cell targets: B10.BR anti-BALB/c (anti-H-2^d) (×); B10.BR anti-C57BL/6 (anti-H-2^b) (+); B10.BR anti-B10.M (anti-H-2^f) (●); C57BL/6 anti-BALB/c (anti-H-2^d) (○); C57BL/6 anti-CBA (anti-H-2^k) (▽); and dm2 anti-BALB/c (anti-H-2L^d) (*). Cytotoxic T cells generated against H-2^b and H-2^f antigens lyse L^d-transfected cells, indicating that there is some crossreactivity between L^d antigen and determinants expressed by H-2^b and H-2^f splenocytes at the cytotoxic T cell level.

nowski et al. (23), Allen et al. (29), and Arnold et al. (30), which indicate that the α1-α2 but not the α3-β_2-microglobulin domain of transplantation antigens carry polymorphic determinants recognized by T cell receptors of cytotoxic T cells.

To determine whether the Q6/L^d molecules carry determinants recognized by other alloreactive cytotoxic T cells and to exclude the possibility of nonspecific crossreactions, we generated killer T cells against splenocytes from mice of H-2^k, H-2^d, H-2^f, and H-2^b haplotypes and tested them on Ltk$^+$ cells and on fibroblasts transfected with the L^d and Q6/L^d genes (Fig. 6). All three target cells were sensitive to the lytic effects of anti-H-2^k killer T cells (B6 anti-CBA), as expected, since L cells express H-2^k antigens. Cytotoxic T cells directed against H-2^d antigens (B10.BR anti-BALB/c and B6 anti-BALB/c) lysed L^d-expressing mouse L cells, while Ltk cells and fibroblasts transfected with Q6/L^d genes were not killed. Cytotoxic T cells generated against H-2^b (B10.BR anti-B6) did not

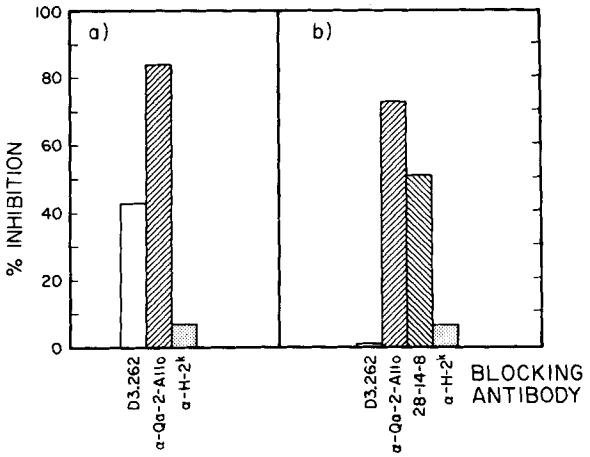

FIGURE 7. Ability of antibodies directed against the Qa-2 subregion–coded molecules to block activity of cytotoxic T cells. Blocking of cytotoxic T cell–mediated lysis with antibodies is shown as percent inhibition of net release of ^{51}Cr from (*a*) B6.K2- or (*b*) Q6/L^d-transfected cloned target cells in the presence of B6.K1 anti-B6.K2 effector cells. Antibodies used for inhibition of lysis included: D3.262 (anti-Qa-2^a) (□); B6.K1 anti-B6.K2 (alloantiserum-specific for Qa-2 region (▨); 28-14-8 (anti-L^d) (▩); and alloanti-H-2^k serum (H-2^d anti-H-2^k) (▣). Percent inhibition = [1 − (net release in the presence of antibody/net release in the presence of control sera)] × 100. Net release from B6.K2 and Q6/L^d target cells in the absence of sera was 36% in both cases.

lyse Ltk cells or cells expressing Q6/L^d molecules. These results show that Q6/L^d molecules are not recognized by cytotoxic T cells with specificities directed against transplantation antigens. Thus, the structural determinants on Q6 hybrid molecules that define T cell receptor specificity are distinct from those found on class I molecules.

Cytotoxic T cells can be used to determine whether the serologically defined Qa-2 molecule and hybrid Q6 molecule share polymorphic determinants. We used an anti-Qa-2 monoclonal antibody and an anti-Qa-2 region alloantiserum to block the Qa-2-specific T cell killing of the various L cell transfectants and spleen cells. The assumption is that antibodies recognizing the same structural regions as the cytotoxic T cells will block killing. The B6.K1 anti-B6.K2 cytotoxic T cells were tested for their lytic effect against B6.K2 (Qa-2^a) target cells (Fig. 7). In the presence of an anti-Qa-2 alloantiserum, the lytic effect was blocked, as previously demonstrated (22). Further, using the anti-Qa-2 monoclonal antibody D3.262, we were able to block a portion but not all of the T cell lytic activity against normal spleen cells (Fig. 7 and reference 22). As a control, we show that this cytotoxic effect was not inhibited by anti-H-2^k sera. When the cytotoxic T cells were tested against the Q6/L^d target cells, we were able to block the cytotoxic effect with the anti-Qa-2 region alloantiserum, B6.K1 anti-B6.K2. The monoclonal antibody D3.262 had no blocking effect. Thus, Q6-encoded determinant(s) recognized by cytotoxic T cells can be blocked by an antiserum directed against the multiple antigenic specificities of the Qa-2 region, but not by a single monoclonal antibody directed against a specific Qa-2 molecule. Clearly, the Qa-2 region encodes a multiplicity of different antigenic determinants that may be

found on distinct class I molecules. Alternatively, D3.262 antibody reacts with the $\alpha 3$ domain of the Q6 molecule not present in the Q6/L^d hybrid.

Discussion

$\alpha 1$-$\alpha 2$ Domain of the Q6 Polypeptide Is Approximately as Closely Related to Homologous Domains in Transplantation Antigens as to Those of Other Qa Antigen. Amino acid comparisons of the $\alpha 1$-$\alpha 2$ domain of six different class I polypeptides (10, 18, 33–40) reveal that 33 differences separate the K^d and L^d $\alpha 1$-$\alpha 2$ domains, whereas 32 differences distinguish the homologous K^b and D^b regions. The Q6 $\alpha 1$-$\alpha 2$ domain differs from those of the K^d and L^d polypeptides by 37 and 39 residues, respectively. The differences are scattered in a 182 amino acid sequence of the $\alpha 1$-$\alpha 2$ domains. Thus, the Q6 antigen is no more K^d-like than L^d-like in its N terminus, and the presence of Q6 polymorphic determinants recognized by 20-8-4 and 34-1-2 antibodies and similar to those found on K^d antigen cannot merely be attributed to a translocation or recombination of a K^d-like gene to Qa-2 region. The Q6 $\alpha 1$-$\alpha 2$ external domain differs from the H-2^b Q10-secreted class I product (39) by 37 residues. No unique amino acid substitutions common to Q6 and H-2^b Q10 and absent from other transplantation antigens could be identified with the exception of a change of aspargine to lysine at position 176 in the $\alpha 2$ region. This change, leading to a loss of glycosylation site, is also present in the HLA B7 allele (41). Its functional significance, if any, is not known. Thus, the coding sequences of the Q6 and H-2^b Q10 genes differ as extensively from one another as either does from those of the transplantation antigens. Perhaps gene conversion events are frequent among the class I genes of the H-2 and Qa/Tla regions and this correction process leads to the rapid divergence and shuffling of the coding sequences (42, 43). One wonders if the structural similarities of the classical transplantation antigens and the Qa polypeptides reflect similar functions of these antigens.

Q6 Gene May Encode CR (Crossreactive) Antigen. The serological and killer T cell analyses described in this paper demonstrated that spleen cells of some strains of mice express Q6 antigens or molecules with crossreacting determinants. Indeed, the Q6 gene product may be the CR antigen recently described by Sharrow et al. (44). This antigen encoded by the Qa-2 region is expressed primarily on peripheral T cells, Ia-positive cells, and bone marrow cells, and reacts with monoclonal antibodies 20-8-4 and 34-1-2. The reactivity could be blocked by B6.K1 anti-B6 serum specific for molecules encoded in the Qa and Tla regions. Therefore, Sharrow et al. (44) suggested that B6.K1 anti-B6 serum and the anti-H-2 monoclonal antibodies detect distinct but spatially related antigenic determinants on the CR antigen. The determinants detected by B6.K1 anti-B6 serum are absent on K transplantation antigens, because the reaction of monoclonal antibodies 20-8-4 and 34-1-2 with K antigens could not be blocked with anti–Qa/Tla region serum. Since Q6/L^d fusion product has a similar pattern of reactivities to the CR, and K^b, and K^d antigens, we subjected it to the same type of inhibition analysis and found that it is indistinguishable by these criteria from CR antigen. Thus, the Q6 sequence may be a structural gene for the CR antigen.

Q6 Gene Product Is Probably a Cell Surface Molecule. If the Q6 gene product

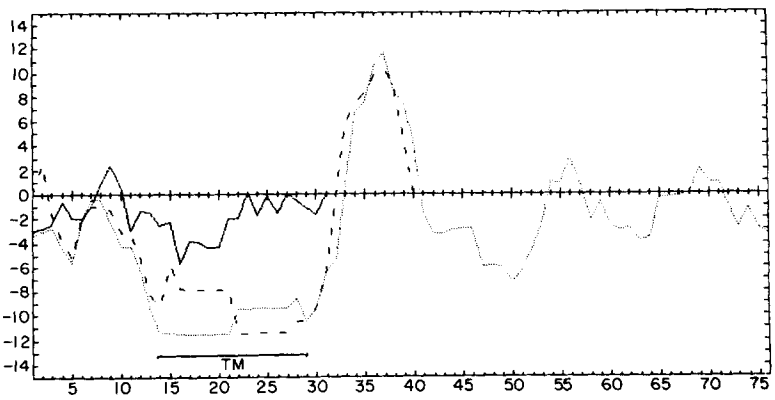

FIGURE 8. Hydrophilicity profiles of class I molecules. Carboxyl-terminal regions of H-2^b Q10 (---), Q6 (— —), and K^d (· · ·) proteins are shown. Hydrophobicity values were calculated by a computer program similar to that of Kyte and Doolittle (46) and written by S. Kent and F. Larsen (personal communication). The positive values correspond to greater hydrophilicity values. The program was run at a span setting of 7. The protein sequence starts with the first amino acid encoded by exon 5. It corresponds to position 275 in the K^d sequence. The solid bar below the profile corresponds to K^d region proposed to span the membrane.

is the CR antigen, it should be an integral membrane protein. Yet the Q6 gene differs from typical class I genes in several features. First, the Q6 gene contains a stop codon located nine nucleotides after the end of the transmembrane coding region. If the Q6 protein is translated, it would be ~40,000 mol wt. Second, there is a charged aspartic acid residue in the middle of the putative transmembrane region. This observation raised the possibility that the Q6 antigen might be secreted, as proposed for the H-2^b Q10 antigen (39, 45). However, inspection of the amino acid hydrophilicity profile of the hypothetical transmembrane regions of the Q6, H-2^b Q10, and K^d polypeptides reveals that the presence of the single charged amino acid in the transmembrane segment of the Q6 antigen does not alter significantly its hydrophobicity (Fig. 8). When the hydropathy of a given segment of a protein (19 residues or longer) averages >1.6, there is a high probability that it will span the membrane (46). The mean hydropathy of the K^d and K^b transmembrane regions, calculated according to the hydrophobicity rules of Kyte and Doolittle (46), is 2.7 and 2.5, respectively, while the corresponding segment of the H-2^b Q10 antigen is 0.5. The transmembrane segment of the Q6 antigen scores an average hydropathy of 2.5. Thus, these calculations suggest that Q6 polypeptide is membrane bound. If that is the case, the anchoring hydrophilic cytoplasm-bound segment of the Q6 antigen would be only four amino acids long, as opposed to ~25 for the transplantation antigens. Recent results (47, 48) demonstrated that transplantation antigens with altered or truncated cytoplasmic domains can still be expressed on the cell surface of transfected fibroblasts and function as targets for killer T cells. When a termination codon is placed in the L^d gene by in vitro mutagenesis, so that the cytoplasmic region is just four residues long, this class I molecule is displayed on the cell surface (49). Hence, Q6 probably is an integral membrane protein.

We have mapped the feature responsible for the absence of Q6 cell surface expression on mouse fibroblasts to the 3′ half of the gene. Several different

mechanisms could explain this effect. It is possible that a regulatory element necessary for the tissue-specific transcription located in the 3′ region of the Q6 gene is inactive in the fibroblasts. Alternatively, the cell surface expression of Q6 molecules may require the interaction of the $\alpha 3$ region or cytoplasmic region with tissue-specific protein(s). Finally, the Q6 gene may be a pseudogene, although this explanation appears highly unlikely: it has an open reading frame throughout, encodes polymorphic determinants expressed on mouse spleen cells, has a functional promoter, and, by sequence criteria, appears to be a cell surface antigen. We are presently trying to distinguish experimentally among the different possibilities. Specific probes for the Q6 gene will be isolated and used in hybridization experiments to test if Q6 gene expression is regulated at the RNA level. Since Q6 DNA is most likely a structural gene for the CR antigen, it will be introduced into T cells to determine whether it is displayed on the cell surface.

Summary

Coding potential of the Q6 gene from the Qa-2^a region of BALB/c Crgl mice was analyzed by a combination of hybrid class I gene construction and DNA-mediated gene transfer. Recombinant genes were created by exon shuffling of the 5′ coding region of the Q6 gene and the 3′ coding region of a gene encoding a transplantation antigen (K^d, D^d, or L^d), or the inverse. Some of these hybrid class I genes were expressed in the transfected mouse fibroblasts (L cells). The hybrid class I molecules encoded by the 5′ end of the Q6 gene and the 3′ end of the L^d gene precipitated as 45,000 mol wt molecules associated with β_2-microglobulin. The expression of the hybrid proteins indicates that 926 basepairs of the 5′ flanking region upstream of the structural Q6 gene contain a promoter that functions as a transcription initiation site in L cells. The 3′ portion of the Q6 gene appears to be responsible for the lack of cell surface expression of the intact Q6 and the hybrid L^d/Q6 genes in mouse fibroblasts. Accordingly, this portion of the Q6 class I gene may play a regulatory role in tissue-specific expression. Serological analyses of hybrid Q6 proteins suggested that Q6 may be a structural gene for CR (H-2 crossreactive) antigen found normally on subpopulations of lymphocytes. If this identification is correct, Q6 gene will define a new category of class I genes encoding ~40,000 mol wt molecules and carrying a characteristic truncated cytoplasmic tail. Analysis of L cells transfected with Q6 hybrid genes demonstrated also that the cytotoxic T cells specific for Qa-2^a region–coded antigens recognize the amino-terminal $\alpha 1$-$\alpha 2$ domain of Q6 fusion products. This recognition can be blocked by anti-Qa-2^a alloantiserum and monoclonal antibodies reactive with the $\alpha 3$-β_2-microglobulin portion of the Q6 hybrids. We propose that the structural requirements for the anti-Qa-2^a cytotoxic T lymphocyte–specific epitopes on target molecules are the same as for anti-H-2-alloreactive cytotoxic T lymphocyte determinants on transplantation antigens and that the mechanism of target recognition is similar in both cases. This interpretation is consistent with the following structural similarities found in both categories of class I molecules: (*a*) K^d and Q6 $\alpha 1$-$\alpha 2$ domains share serologically defined epitopes. (*b*) Cytotoxic T lymphocyte–specific and antibody-specific determinants on Q6- and H-2-defined $\alpha 1$-$\alpha 2$ domains are not distorted in hybrid proteins carrying different polymorphic $\alpha 3$-β_2-microglobulin carboxyl

termini. (*c*) Amino acid comparisons between H-2 and Qa molecules indicated that the two groups are almost as closely related to each other as the individual members within each group.

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